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Up-Regulation of Amphotrophic Retroviral Receptor Expression in Human Peripheral Blood CD34+ Cells

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Retroviral-mediated gene transfer into hematopoietic stem cells provides the only means of stable transduction of these cells and their progeny for use with a variety of potentially therapeutic genes. Expression of the Moloney amphotropic retroviral receptor—pit-2 or GLVR-2—is critical to the recognition and entry of Moloney leukemia virus-derived viruses into human target cells such as CD34+ hematopoietic cells. GLVR-2 functions as a sodium-dependent phosphate transporter as well as a receptor. We have previously shown that the expression of the murine homologue of the amphotropic receptor *Ram*1, also a phosphate transporter, is developmentally regulated in murine hematopoietic fetal liver cells. We also demonstrated that culture of murine fetal liver cells in phosphate-free (PO_4-free) medium increases levels of receptor mRNA and makes murine fetal liver cells susceptible to Moloney amphotropic viral gene transfer. We now examine the effect of culture conditions on the expression of GLVR-2 in human CD34+ cells. In this report, we demonstrate that there is a 2–3 fold increase in GLVR-2 mRNA levels in CD34+ cells after 3 days in culture with interleukin 3, interleukin 6, and stem-cell factor. In addition, the use of PO_4-free medium increases expression of GLVR-2 an additional 2-fold in these cells during this time. These results indicate that GLVR-2 expression can be up-regulated on these cells, and may permit improved retroviral gene transfer efficiencies. Am. J. Hematol. 61:243–253, 1999. © 1999 Wiley-Liss, Inc.

Key words: retroviral; amphotropic receptor; gene transfer; gene therapy

INTRODUCTION

Retroviral-mediated gene transfer into hematopoietic stem cells provides the only means of stable transduction of these cells and their progeny for use with a variety of potentially therapeutic genes [1,2]. Stem-cell transduction has been achieved in mice for the human MDR gene and the human β-globin gene by targeting the ecotropic (MLV) retroviral receptor on these cells [3–7]. An amphotropic receptor must be targeted to transduce human cells. The human amphotropic receptor—GLVR-2 or pit-2—is a member of a group of transmembrane proteins which function as phosphate transporters, as well as the receptors for envelope proteins of certain retroviruses. Sufficient GLVR-2 expression on human CD34+ cells is required for adequate transduction of these cells, if this receptor is to be targeted by retroviruses.

Several lines of evidence suggest that the level of rodent or human amphotropic retroviral receptor expression correlates with the ability of MLV to gain entry into target cells. Improved retrovirus-mediated transduction efficiencies have been demonstrated when an adenoviral vector has been used to overexpress *Ram*1, the murine homologue of the amphotropic retroviral receptor in a number of cell types [8]. This effect was greater for cell types with low or absent basal *Ram*1 expression than for cell types with high basal *Ram*1 expression. In another study of murine cells, it was concluded that the low level of amphotropic receptor expression is responsible for the lower transduction efficiency of amphotropic retrovirus-

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TABLE I. Primers Used for Generation of Competitive DNA Fragments and for Use in Competitive PCR

Primer	Sequence (5′–3′)
β_2M-0	sense: GCG TAC TCC AAA GAT TCA GG
β_2M-2	anti-sense: ATC TTC AAA CCT CCA TGA TG
GL2-757	sense: AAG GAA GAC CCT GTT CCC A
2LG-1413	anti-sense: GGT GTA GCA GGT GTA ACT G
GL2-DEL	sense: AAG GAA GAC CCT GTT CCC A TA GCA ATC AAT GTC TTT TCC ATC ATG TAC AC

es, when compared to that of ecotropic retroviruses [9]. Also, culture of rat fibroblast cells in PO_4-free medium has been noted by others to increase *Ram*1 message [10].

We have previously reported that *Ram*1 is developmentally regulated during mouse hematopoiesis, being expressed at low levels on murine hematopoietic fetal liver cells (FLC), and at higher levels on adult mouse bone marrow cells [11]. In addition, *Ram*1 expression is low on murine hematopoietic cell populations enriched for stem cells [12]. In these studies, we also showed that in these murine cells, low-phosphate (PO_4) medium induced increased levels of *Ram*1 message, sufficient to render previously resistant murine FLC susceptible to transduction with an multiple-drug resistance (MDR)-containing amphotropic retrovirus [12].

It is probable that high-level expression of GLVR-2 on human CD34+ cells is critical for optimal transduction of these cells, if this receptor is targeted by the retroviral envelope. One study reported low levels of amphotropic receptor mRNA on human CD34+ cells, especially in CD34+/CD38− progenitor cell subpopulations [9]. In another, GLVR-2 overexpression in HeLa cells and squamous carcinoma cell lines improved amphotropic retrovirus mediated gene transfer [13]. These observations and our promising results in up-regulating *Ram*1 on murine cells encouraged us to investigate GLVR-2 expression in cultured CD34+ hematopoietic precursor cells, and its potential up-regulation in PO_4-free medium on receptor expression. We report here GLVR-2 levels on human CD34+ cells by using a sensitive competitive reverse transcription (RT)-polymerase chain reaction (PCR) technique, a reliable method of quantifying low levels of DNA and RNA in clinical and experimental samples [14]. We show that GLVR-2 expression of CD34+ cells increases 2–3-fold during 3 days of culture with growth factors, and increases an additional 2-fold in low-PO_4 medium during this time. The results suggest that up-regulation of GLVR-2 expression on these cells is feasible and may improve gene transfer efficiencies into human hematopoietic cells.

Materials and Methods

CD34+ Cell Selection and Analysis

Peripheral blood samples were obtained from patients undergoing apheresis prior to autologous bone marrow transplantation. Peripheral blood stem cells (PBSC) containing CD34+ cells were obtained after mobilization with 9 days of 300 mg (s.q.) recombinant G-CSF (Amgen) alone, or cyclophosphamide 1.5 gm/m^2 i.v. and 9 days of G-CSF. Red blood cells were eliminated from PBSC harvests by a 5 min lysis in 1× hemolysis buffer (0.16 M ammonium chloride, 0.01 M sodium bicarbonate, 0.01 M sodium ethylenediaminetetraacetic acid). The resulting nucleated cell fraction was washed in 1% bovine serum albumin in phosphate-buffered saline (Sigma) and up to 2×10^8 cells were applied to a Cellpro CEPRATE column [15], and CD34+ cell fractions collected; aliquots were taken for FACS analysis and clonogenic assays. Flow cytometry was performed as previously described [16]. Between 70% and 95% of the cells were CD34+ as assessed by this method. Immediately after isolation, viability of cells was measured by trypan blue staining.

Cell culture

CD34+ cells were cultured on day 1 in 25 ml of Iscoves' modified Dulbecco's medium (IMDM, Life Technologies, Bethesda, MD) supplemented with 20% fetal calf serum (FCS, Gemini Bioproducts, Calabasas, CA), 1% penicillin-streptomycin (Life Technologies), recombinant human interleukin-3 (IL-3), interleukin-6 (IL-6), and stem-cell factor (SCF) each at 50 ng/ml (Amgen Biologicals, Thousand Oaks, CA) before further evaluation [17]. Cells were cultured in a humidified incubator with a 5% CO_2 atmosphere at 37°C. On day 1, an aliquot was taken for RNA extraction and the remaining CD34+ cells were split into two equal fractions. One fraction was maintained in the supplemented medium as described above (referred to as normal-PO_4 medium). The other fraction was washed twice in phosphate-buffered saline and subsequently cultured in PO_4-free medium. PO_4-free medium consisted of DME medium (Life Technologies), supplemented with 20% dialyzed FCS (Life Technologies), 1% penicillin-streptomycin (Life Technologies), recombinant IL-3, IL-6, and SCF each at 50 ng/ml (Amgen). Cell aliquots ranging from 1×10^6 to 1.3×10^7 cells were taken for RNA extraction every 24 hr, and the remaining culture volume was restored to 25 ml by adding the required volume of appropriate media. Viability of all aliquots was assessed by trypan blue staining.

RNA Extraction

Total RNA was isolated from either fresh cell pellets or from cells flash frozen and stored at −86°C by using a commercial silica membrane spin column kit (RNeasy, Qiagen, Chatsworth, CA). Pellets were homogenized in 0.35 ml of lysis buffer by using a 1 ml tuberculin syringe with a 22 gauge needle. Samples were applied to the columns, washed, and eluted as per the manufacturer's

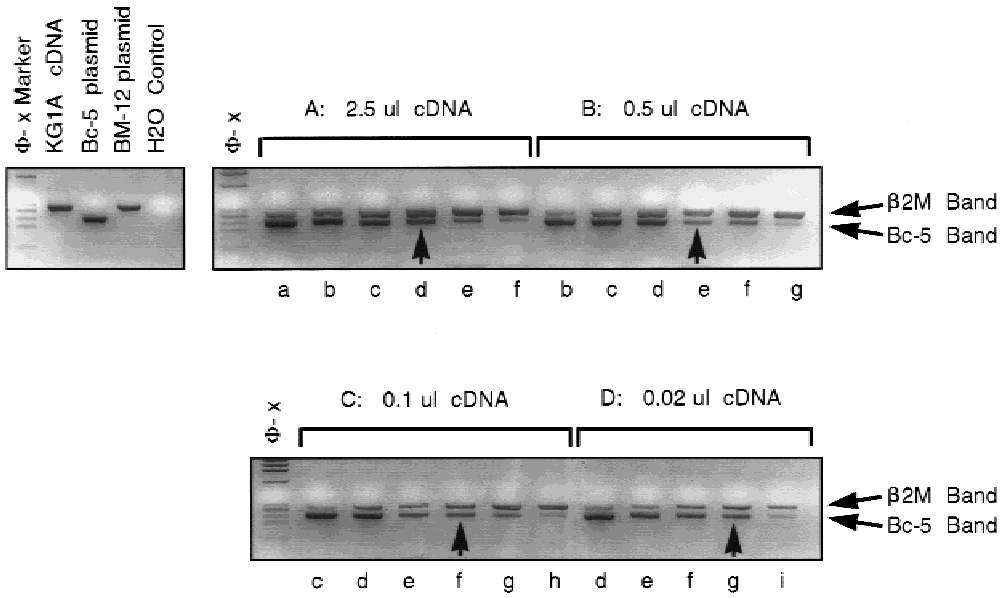

Fig. 1. Validation of β2-microglobulin (β2M) quantitation by competitive PCR. Aliquots containing a constant amount of KG1a cDNA were amplified by PCR together with serial dilutions of competitor DNA plasmid Bc-5. Four different amounts of cDNA were subjected to quantitation by competitive PCR: A, 2.5 μl cDNA; B, 0.5 μl cDNA (1/5 dilution of A); C, 0.1 μl cDNA (1/5 dil. of B); and D, 0.02 μl cDNA (1/5 dil. of C). Each of these sample volumes of cDNA was amplified in the same PCR tube with one of the following panel of serially diluted competitor DNA. Dilution a represents a 10^{-3} μg/ml stock solution of competitor plasmid Bc-5. In order, dilutions b through i were generated by serial 1/4 dilution of dilution a. Competitor DNA concentrations used were thus: a, 10^{-3} μg/ml; b, 2.5×10^{-4} μg/ml; c, 6.25×10^{-5} μg/ml; d, 1.6×10^{-5} μg/ml; e, 3.9×10^{-6} μg/ml; f, 9.8×10^{-7} μg/ml; g, 2.4×10^{-7} μg/ml; h, 6.1×10^{-8} μg/ml; and i, 1.5×10^{-8} μg/ml. The point at which the competitor concentration is equal to that of the sample, the point of equivalence and can be roughly determined by visual inspection of the above gels as the point at which sample and competitor DNA produce bands of equal density. For A, 2.5 μl cDNA, the equivalence point appears to be at a competitor dilution d as indicated by the arrowhead. Similarly, for B, 0.5 μl cDNA, the equivalent competitor dilution is e, for C, 0.1 μl cDNA, the equivalent competitor is f, and for D, 0.02 μl cDNA, the equivalent competitor is g. Controls KG1a cDNA and BM-12 plasmid (containing a cDNA derived β2M insert) demonstrate the size of the wild-type β2M amplimer; Bc-5 competitor DNA alone produces a smaller band, H_2O is a water negative control.

protocol. Forty units of RNase inhibitor (Stratagene, La Jolla, CA) were added to each eluate prior to further analysis.

Generation of Competitive and Control DNA

For each sample, up to 1 mg of total RNA was reverse transcribed in a total volume of 50 μl by using a commercial RT-PCR kit (Stratagene). Resulting complementary DNA (cDNA) samples were stored at −20°C until further use. Table 1 lists primer sequences used in the generation of competitive and control DNA. Primers for β2-microglobulin (β2M-0 and β2M-2) were chosen from the published sequence of the β2M gene [18]. cDNA from the human hematopoietic progenitor cell line KG1a was subjected to PCR with primer pair β2M-0/β2M-2. PCR conditions for the β2M-0/β2M-2 primer pair were: $[MgCl_2]$ = 2.0 mM, 95°C for 30 sec, anneal at 56°C for 30 sec, extend at 72°C for 45 sec for 30 cycles, followed by a final extension period at 72°C for 5 min. The resulting 319 base-pair (bp) β2M piece was purified from low-melting-point agarose, and subcloned into plasmid pCR-2.1 by using the Original TA-Cloning Kit (Invitrogen, La Jolla, CA), resulting in plasmid BM-12. In order to generate a suitable piece of competitor DNA, murine NIH-3T3 cDNA was subjected to PCR with primer pair β2M-0/β2M-2 under less stringent conditions. Of several nonspecific bands seen, one was chosen because it was slightly smaller than the 319 bp human β2M PCR product. Repeated amplification by PCR resulted in primer binding sites with complete homology to β2M-0 and β2M-2. A competitor plasmid, Bc-5, was generated by subcloning this 247 bp piece into pCR-2.1, and used for quantitation of β2M.

Primers GL2-757 and 2LG-1413 were chosen from the published sequence of the GLVR-2 cDNA sequence [19]. Human hematopoietic progenitor cell line KG1a cDNA was subjected to PCR with primer pair GL2-757/2LG-1413. PCR conditions for the primer pair GL2-757/2LG-1413 were: $[MgCl_2]$ = 2.5 mM, 95°C for 30 sec, anneal at 54°C for 30 sec, extend at 72°C for 45 sec for 35 cycles, followed by a final extension period at 72°C for 5 min. The 657 bp GLVR-2 piece was gel-purified

Figure 2

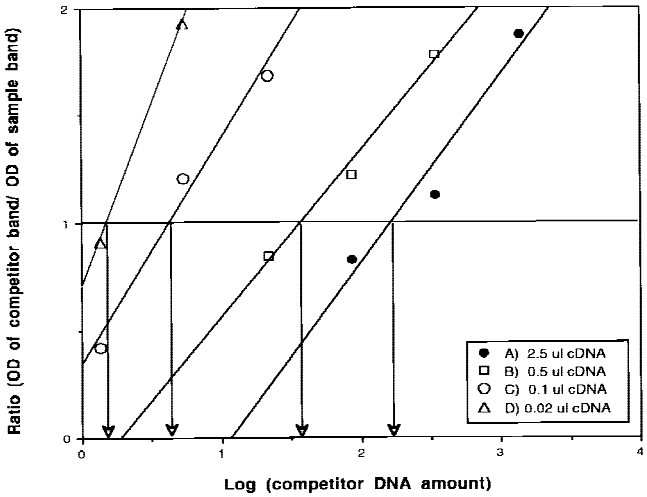

Fig. 2. Analysis of β2M concentrations. For each of the cDNA test samples A–D in figure 1, equivalence between sample and competitor occurs where the density of bands they produce is equal. The point of equivalence is where the ratio of competitor band optical density (OD) to sample band OD is 1 (indicated by the horizontal line at 1 in Fig. 2.). The bands shown in figure 1 were subjected to optical densitometry. The ratio of OD of the competitor band to the OD of the sample band was calculated for each competitor dilution tested and the three ratios closest to the point of equivalence plotted against $\log_{10}$ (competitor concentration × 10^8). From figure 1, the ratios from A, dilutions c, d, and e are plotted; from B, dilutions d, e, and f; from C, dilutions e, f, and g; and from D, dilutions f, g, and i. A "best-fit" line was computed by regression analysis and plotted above. Sample concentrations determined in this manner are represented by the point of equivalence and indicated above with arrowheads on the horizontal axis. They were determined by substituting $y = 1$ in the equation of the best fit line equation ($y = mx + c$) obtained by regression analysis.

and subcloned into pCR-2.1 as above, resulting in plasmid GL-3. A 47 bp oligonucleotide (GL2-DEL) was designed to generate a specific 5′ deletion, while maintaining a desired 5′ PCR primer site in a single PCR run. The 3′ 32-base pairs of oligo GL2-DEL are homologous to the GLVR-2 cDNA downstream (bp 884-915 of the cDNA sequence) from the 5′ primer binding site (GL2-757), while the 19 5′ nucleotides of oligo GL2-DEL are the same as the 5′ primer GL2-757. By subjecting GL-3 to PCR with primer pair GL2-DEL/2LG-1413, a fragment was generated with a deletion at the 5′ end of the GL-3 insert, while generating a new 5′ end with perfect homology to 5′ primer GL2-757. This shorter DNA fragment was inserted into pCR-2.1, generating plasmid GL-del-4, our competitor DNA for quantitation of GLVR-2. Identity, length, and fidelity of primer binding sites were confirmed for all of the above plasmid inserts by direct, bi-directional DNA sequencing.

TABLE II. Competitive PCR Test on Dilutions of cDNA*

Volume of cDNA added to PCR tube (ml)	Amount of β2M detected (arbitrary units)	Experimentally observed dilution factor	Expected dilution factor (⅕ serial dilutions)	Error from expected (%)
A) 2.5	250	1	1	N/A
B) 0.5	44	5.7	5	+13%
C) 0.1	7.4	5.9	5	+18%
D) 0.02	1.7	4.4	5	−12%

*Calculation of β2M concentrations: Amount of β2M (derived in Fig. 2) in sample dilutions A through D are listed in arbitrary units. Because sample dilutions B, C, and D were generated by serial ⅕ dilution of A, the experimentally observed concentration should also decrease by a factor of ⅕ for each successive dilution. Experimentally observed dilution factors were $⅕_{5.7}$ for B, $⅕_{5.9}$ for C, and $¼_{4.4}$ for D and are close to the expected dilution factor of ⅕. Errors from the expected values ranged from −12% to +18%.

Competitive PCR

Competitive PCR was performed analogous to that used to quantitate bcr-abl rearranged mRNA in blood samples of patients with chronic myelogenous leukemia [20]. Optical densitometry would have been an inadequate technique for determining the low amounts of starting total RNA obtained from CD34+ cells. We used quantitative RT-PCR for the housekeeping gene β2M as a control for correcting GLVR-2 expression for the amount of starting total RNA as described [21]. Sample cDNA was diluted prior to addition to PCR reactions to minimize errors associated with pipetting small sample volumes. cDNA for β2M quantitation was diluted 1:5 prior to analysis; cDNA for GLVR-2 analysis diluted 1:2. Subsequent calculations take these dilutions into account. Ten μl of these sample dilutions were then used for each competitive PCR reaction.

Whole plasmid DNA was used as competitor DNA. 1.0 μg/ml stock solutions of competitive DNA plasmids Bc-5 and GL-del-4 were diluted 1/1,000 and termed dilution a. Subsequent 1:4 serial dilutions were used as competitor DNA dilutions in the quantitation of β2-microglobulin and GLVR-2 cDNA, respectively. Serial plasmid dilutions were labeled a through i and ranged in concentration from: a, 10^{-3} μg/ml to i, 1.5×10^{-8} μg/ml. Generally, β2M was relatively abundant and found its equivalent concentration in dilutions b through e, whereas GLVR-2 was a rarer transcript with its equivalent among dilutions e through i, as detailed in the results. A fixed amount of experimental sample cDNA (10 μL of the above cDNA dilutions) was added to each of five successive competitor DNA dilutions in separate PCR tubes, and subjected to amplification using appropriate PCR conditions. A 15 μL aliquot of the PCR products was subjected to electrophoresis in a 4% agarose gel containing ethidium bromide. The resulting gel was pho-

TABLE III. Competitive PCR Test on Dilutions of GL-3 Plasmid*

Dilution of GL-3 plasmid used as sample (ug/ml)	Amount of GL-3 used as sample (arbitrary units)	Experimentally determined amount of GL-3 (arbitrary units)	Error from expected (%)
C) 6.25×10^{-5}	1250	955	−24%
E) 3.9×10^{-6}	78	104	+33%
G) 2.4×10^{-7}	4.9	4.2	−14%

*Calculation of GLVR-2 concentrations. Experimentally determined amounts of GL-3 (Figs. 3 and 4) are listed and compared to the actual amount of GL-3 used. Errors from expected for the three determinations are given in %, and range from −24% to +33%.

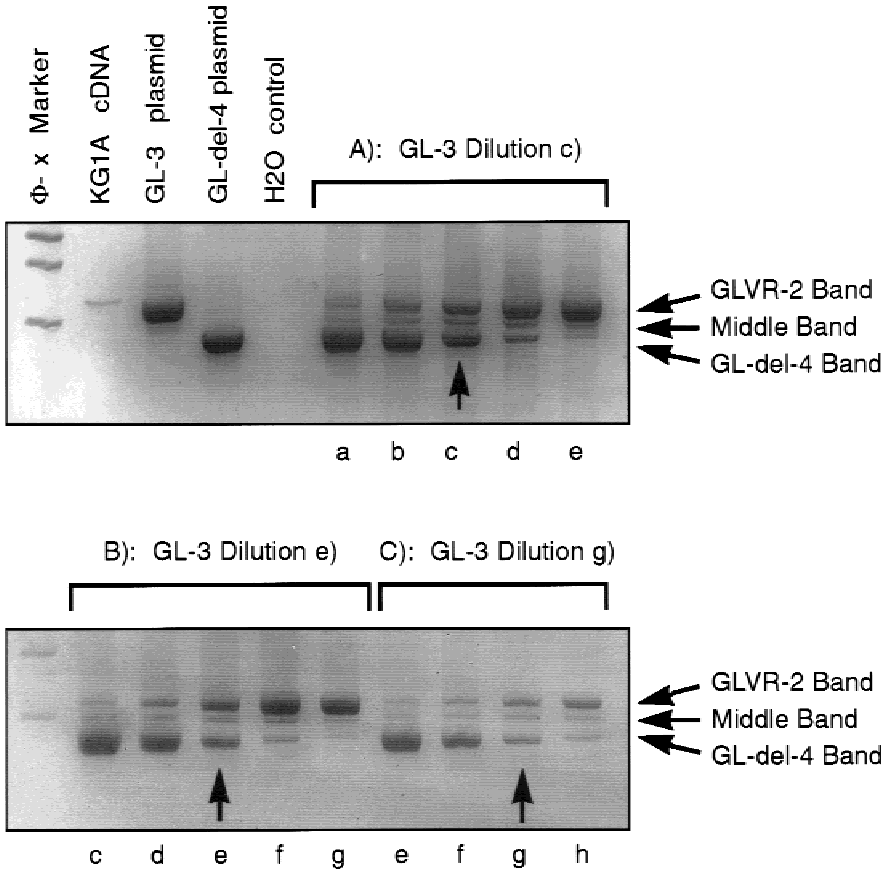

Fig. 3. Validation of GLVR-2 quantitation by competitive PCR. The validity of competitive PCR analysis in determining the concentration of GLVR-2 DNA was tested on dilutions of known concentration of plasmid GL-3 (containing a cDNA derived GLVR-2 fragment). Sample (GL-3) and competitor (GL-del-4) DNA were diluted as described above for plasmid Bc-5 (Fig. 1). Sample (GL-3) dilutions tested were: c, 6.25×10^{-5} μg/ml; e, 3.9×10^{-6} μg/ml; and g, 2.4×10^{-7} μg/ml. These samples were co-amplified in the presence of an appropriate range of competitor DNA (GL-del-4) dilutions as indicated. By inspection of the gel the points of equivalence for GL-3 dilution c appears to be competitor dilution c; similarly, GL-3 dilution e appears equivalent at competitor dilution e, and GL-3 dilution g appears equivalent at competitor concentration g, as expected. Because, in this case, the competitor DNA is the result of a deletion of the wild-type amplimer, the sample and competitor are homologous over the entire length of the competitor DNA. Heterologous annealing between sample and competitor DNA has been reported [10], and is assumed to result in the small additional band, termed "middle band," here. Controls KG1a cDNA and GL-3 plasmid (containing a cDNA derived GLVR-2 insert) demonstrate the size of the wild-type GLVR-2 amplimer; GL-del-4 competitor DNA alone produces a smaller band. H_2O is a water negative control.

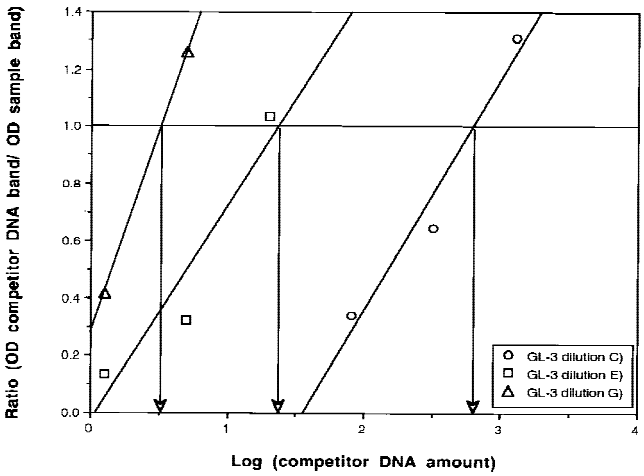

Fig. 4. Analysis of GLVR-2 concentrations. In order to arrive at a calculated point of equivalence for each sample determination, optical densitometry was used as above in figure 2. The ratio of OD of competitor to sample bands was plotted against $\log_{10}$ (competitor concentration × 10^8) for the three points closest to the point of equivalence were plotted as in figure 2. Regression analysis using these three ratios was again used to arrive at a "best fit" line in the form of $y = mx + c$. By substituting 1 for y (by definition the point of equivalence, indicated here by a horizontal line) the calculated sample concentration was determined, and is marked above by the arrowheads.

tographed with Polaroid Type 655 film, which produces a positive print as well as a translucent negative. The optical densities of the sample and competitor bands recorded on the negative were measured using a Molecular Dynamics Computing Densitometer, Model 300A with Image Quant (v4.2) software. Because ethidium bromide binding to DNA is proportional to the length of DNA pieces, the molar amount of shorter DNA pieces (competitor bands here) are underrepresented by ultraviolet-induced ethidium bromide luminescence. This was corrected for as described in [22]—by using the equation ODc = OD (wild-type length/deleted length) where OD is optical density of the shorter band, and ODc the corrected optical density. All calculations were performed by using Microsoft Excel and its data analysis tools.

RESULTS

Competitive PCR

Plasmids containing inserts of β2M and GLVR-2 cDNA fragments were useful not only as positive PCR controls, but also allowed us to make known dilutions of plasmid which were used to test and validate our method of competitive PCR. Known serial dilutions of cDNA derived from KG1a or of plasmid DNA BM-12 and GL-3 were subjected to quantitation by co-amplification with a serially diluted panel of competitive DNA (competitor

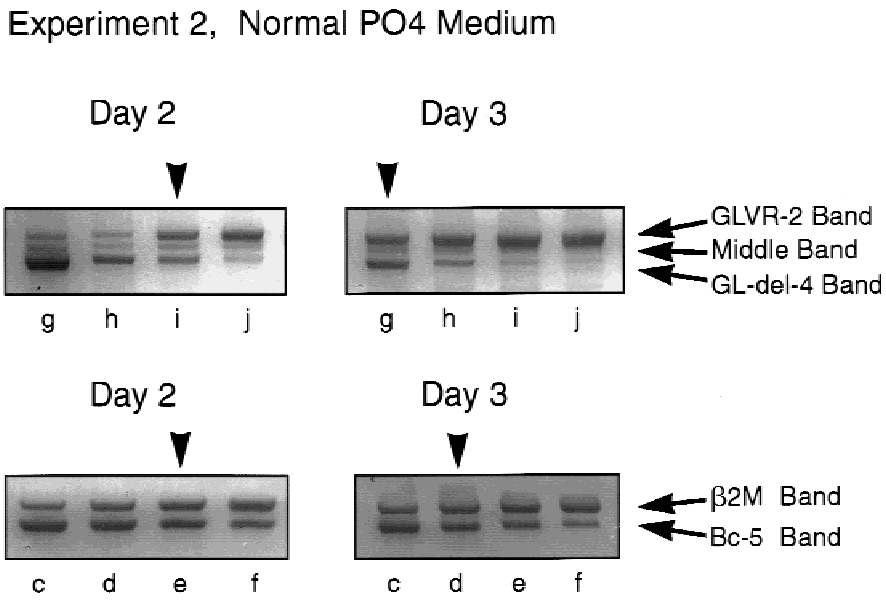

Fig. 5. Quantitation of relative GLVR-2 concentration in CD34+ cells over time. Gels of experiment 2 competitive PCR analysis at two consecutive time points, 2 days (left) and 3 days (right), in normal-PO_4 medium are shown. By gross inspection GLVR-2 expression at 2 days (top left panel) finds its equivalent competitor concentration at competitor dilution i, as indicated by the arrowhead. At 3 days, (top right) GLVR-2 levels find their equivalent in competitor dilution g. Internal control β2M expression in the same samples are shown in the lower panels. At 2 days, β2M levels find their equivalent in competitor dilution e (bottom left panel), at 3 days, it is at competitor dilution d (bottom right panel), indicating a greater amount of starting total RNA in the 2 day sample. Normalization of GLVR-2 levels against internal control β2M, shows higher normalized GLVR-2 levels at 3 days in culture.

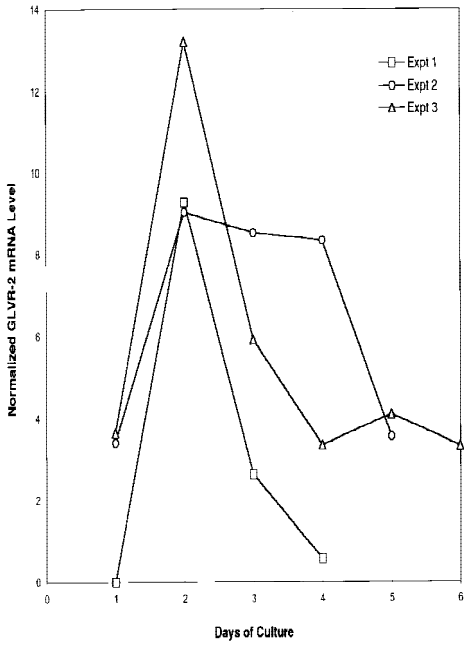

Fig. 6. Time course of GLVR-2 expression in normal phosphate medium. Normalized GLVR-2 mRNA levels in cultured CD34+ cells were determined every 24 hr after isolation in each of three separate experiments. To correct for varying amounts of total RNA, β2M and GLVR-2 mRNA levels were determined for each sample. The ratio of GLVR-2− divided by β2M mRNA levels for each sample is the normalized GLVR-2 mRNA level, and is given in arbitrary units. On day 1, normalized GLVR-2 levels are low, subsequently rising to a peak on day 2 of culture, followed by a more gradual decline in GLVR-2 levels. The pattern of GLVR-2 expression over time is similar in the three experiments.

plasmids Bc-5 and GL-del-4, respectively). At one of the sample/competitor combinations, the starting template DNA concentrations are approximately equal, and when amplified, the resulting sample and competitor bands are of approximately equal intensity (Fig. 1, for example). At this "point of equivalence," the ratio of the competitor band OD to sample band OD is equal to one. To arrive at the competitor and, hence, the sample concentration at the point of equivalence, for each series of sample and competitor combinations, the ratio of competitor to sample band OD was plotted against $\log_{10}$ (competitor concentration), as shown in figure 2, for example. The regression curve in the form $y = mx + c$ was calculated by using the three data points closest to the point of equivalence. By definition, at equivalence, the ratio of OD's at the point of equivalence is 1. Substituting 1 for y allows a mathematical determination of the competitor, and thus sample concentration at equivalence.

To test the relative accuracy of β2M quantitation by competitive RT-PCR, four different dilutions A, B, C, and D of KG1a cDNA were made by serial 1:5 dilution (Fig. 1). Each cDNA concentration was tested against each of six consecutive competitor DNA dilutions. After optical densitometry, the ratio of competitor to sample optical density was utilized to calculate the point of equivalent concentration between sample and competitor. Plots of this ratio against competitor DNA amount are shown in figure 2. Values for β2M concentration in the test dilutions A through D are listed in Table 2. The experimentally observed dilution factors (1:5.7, 1:5.9, and 1:4.4) were close to the actual dilution factor used (1:5).

In an analogous fashion, control plasmid GL-3, containing the sample GLVR-2 insert (657 bp) was used as a standard to test the accuracy of GLVR-2 quantitation by competitive PCR. In the analysis of GLVR-2, a faint third "middle band" is noted midway between the sample and competitor bands. Because, in this case, the competitor DNA is the result of a deletion of the wild-type amplimer (the GL-3 GLVR-2 insert), the sample and competitor are homologous over the entire length of the competitor DNA. Heterologous annealing between sample and competitor DNA has been reported [14], and is assumed to be the cause for the small additional band termed "middle band" here. Half of the optical density of the middle band was ascribed to, and added to the values of each of the sample and competitor bands, respectively, prior to further mathematical analysis (Table 3).

Competitive PCR of dilutions c, e, and g of GL-3 was performed (Fig. 3). As before, regression analysis of the ratios of competitor to sample band OD's yielded lines (Fig. 4) that intersected at $y = 1$ (equivalence point). Numerical results for experimentally determined amounts of GL-3, given in arbitrary units, closely correspond to the actual amount of GL-3 used for testing (Table 3). In a total of nine such GLVR-2 determinations, the error from expected ranged from +58% to −52%, considerably less than one dilution factor of competitor DNA. This compares well with the accuracy of competitive PCR reported by others [20].

Isolation and Viability of CD34+ Cells

CD34+ cells were isolated by affinity column, and efficiency of purification determined by FACS. Serial analysis of cell viability by trypan blue staining of CD34+ cells in normal-PO_4 and PO_4-free media demonstrated generally lower cellular viability in the PO_4-free medium. The viability percentages ranged from 80% to 95% live cells in normal-PO_4 medium, but were as low as 55% in PO_4-free medium, although most values were in the 65% to 85% range. Cell viabilities were somewhat lower in experiment 2 in both types of media, compared to those in experiments 1 and 3 (see below).

Time Course of Amphotropic Receptor Expression

In three experiments, CD34+ enriched cells were cultured in normal-PO_4 as well as PO_4-free medium in the

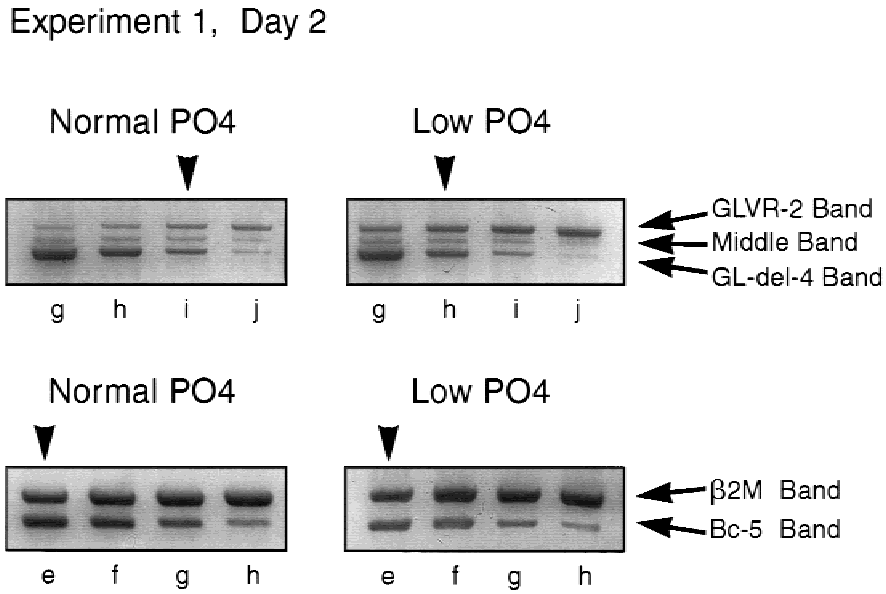

Fig. 7. Quantitation of the relative expression of GLVR-2 in normal-PO_4 and PO_4-free media. Results of experiment 1 competitive PCR analysis in normal-PO_4 (left) and PO_4-free media (right) at day 2 of differing phosphate exposure are shown above. Visual inspection reveals GLVR-2 expression in normal-PO_4 medium (top left panel) to be equivalent at competitor dilution i. In PO_4-free medium GLVR-2 levels are equivalent at competitor dilution h reflecting a higher GLVR-2 expression in this medium. Normalized against β2M expression, this difference persists because the equivalent for β2M expression is equal at competitor dilution e.

presence of IL-3, IL-6, and SCF. CD34+ cells cultured in normal-PO_4 medium permitted analysis of GLVR-2 expression over time in standard culture conditions we have used for hematopoietic precursor cells [16,17]. Figure 5 shows the competitive PCR results from a typical experiment: experiment 2 at days 2 and 3 of culture in normal-PO_4 medium. The point of equivalence for GLVR-2 expression can be seen to be at a higher competitor concentration at day 3 compared to day 2, (concentration i to g) for GLVR-2 as compared to e to d for β2M). This difference persists after normalization against β2M expression which is close to equal in these two samples. This reflects a rise in GLVR-2 expression during this period. Figure 6 shows the normalized GLVR-2 expression in normal-PO_4 medium over time in three experiments using the same analysis as is detailed in figure 5 to obtain each individual point on this figure. On day 1, GLVR-2 mRNA expression is low, but subsequently rises to a peak on day 2 and drops off to lower levels over the next few days in culture. A similar rise and fall of GLVR-2 expression was noted in each of the three experiments (Fig. 6).

Effect of CD34+ Cell Culture in Phosphate-Free Medium

On day 1, half the cells were kept in normal media, and half placed in PO_4-free media. Figure 7 shows the competitive PCR of a typical experiment: experiment 1 on day 2 in normal-PO_4 medium and PO_4-free medium. GLVR-2 expression is higher in PO_4-free medium, than in normal-PO_4 medium. This difference persists after normalization for β2M expression. Figure 8 summarizes the relative levels of GLVR-2 expression in normal and PO_4-deficient medium over time. GLVR-2 mRNA levels increase and peak at approximately the same time, day 2 to 3 of culture in both types of medium. The level of GLVR-2 expression is higher in PO_4-free medium than in normal-PO_4 medium at days 2 and 3 in all three experiments. The only exception is in experiment 1 on day 2 of culture, in which GLVR-2 expression is higher in normal-PO_4 medium. Even in this experiment, there is greater GLVR-2 expression on day 3 in PO_4-deficient medium than normal media. After day 3 of culture, GLVR-2 levels tend to decline to similar low levels in both types of medium.

DISCUSSION

CD34+ cells are important targets for therapeutic gene insertion into human hematopoietic progenitor and stem cells because of their accessibility, and their potential for specific selection and expansion of desired clones. Retroviral gene therapy in clinical trials targeting GLVR-2 on CD34+ cells, however, has been limited to date by the efficiency of gene transfer achieved into transduced cells capable of marrow reconstitution, although progenitors as measured in methylcellulose have been efficently targeted [2,16,23–25]. We feel that expression of GLVR-2

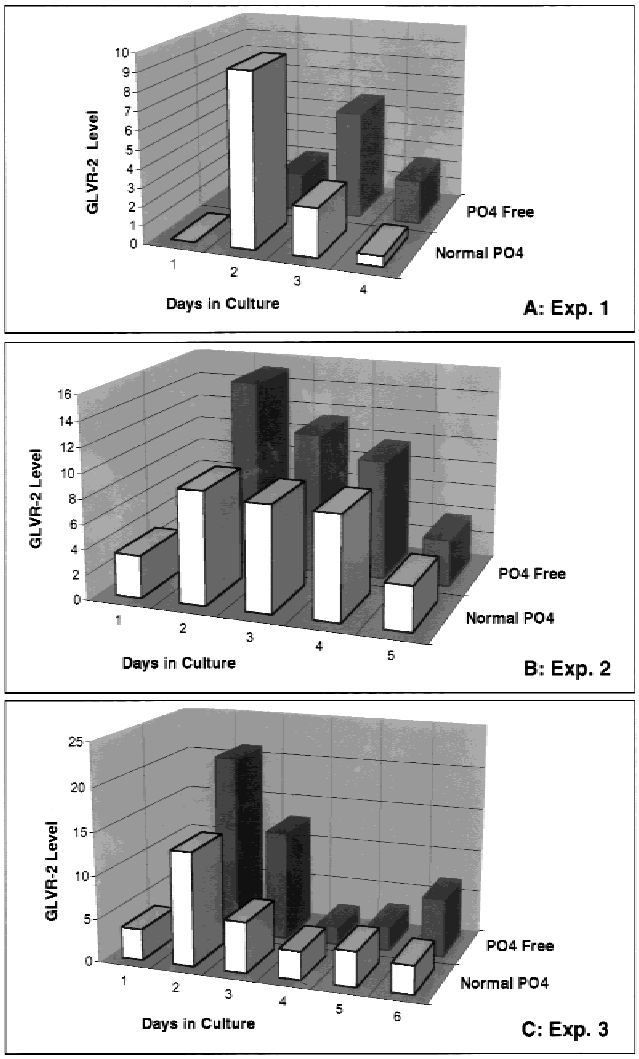

Fig. 8. Summary of relative expression of GLVR-2 mRNA in CD34+ cells in culture. Normalized GLVR-2 mRNA levels of CD34+ cells in normal-PO_4 and in PO_4-free media are compared in each of three experiments. White bars (in front) represent normalized GLVR-2 levels in normal-PO_4 medium and are the same data presented in figure 6. Dark bars (in the rear) show normalized GLVR-2 mRNA levels of CD34+ cells cultured in PO_4-free medium. Peak GLVR-2 expression is reached at the same time in both types of media at day 2. In PO_4-free medium, however, the level of GLVR-2 expression is generally higher during days 2 and 3 of culture, and rises to up to twice the level seen in normal-PO_4 medium.

on CD34+ cells capable of marrow repopulation limits the ability of these cells to be transduced and expanded in vivo.

Results of viral binding studies [26], overexpression of amphotropic receptors on target cells [8,13], and measurements of receptor RNA levels [8,9,12,13,27] support this hypothesis. Since this work was completed and preliminary results presented [28], two papers have measured human amphotropic receptor expression [29,30]. In one of these reports, cell sorting with an antibody to the receptor is used to show retroviral binding is increased in mobilized peripheral blood CD34+ cells as compared to marrow cells, and is correlated with transduction efficiency [30]. We have also previously reported a correlation between increased murine amphotropic receptor expression on target cells and increased transduction efficiency [12].

In our previous experience, optimal retroviral transduction of CD34+ cells occurs after 3 days of culture [16,17,31]. We observed a 2–3-fold increase in GLVR-2 message at about this time, and a fall thereafter. The time course of this rise and fall in GLVR-2 expression appears to be similar in normal medium and in PO_4-free medium. We also observed an additional 2-fold increase in GLVR-2 expression in PO_4-free medium above that seen in normal-PO_4 media. This enhancement of amphotropic receptor expression is most pronounced at days 2–3 in PO_4-deficient media. Although this trend was consistent, some variability in normalized GLVR-2 expression was seen, and the small number of experiments does not allow detailed statistical analysis. After more than 3 days in culture, GLVR-2 levels tended to approach similar lower levels in both types of media, presumably as a result of other intracellular metabolic processes limiting amphotropic receptor expression. Viability of CD34+ cells was also somewhat lower in PO_4-deficient medium than in normal media.

We used commercially available dialyzed serum for the PO_4-free media in our studies. Although we ascribe the differences in GLVR-2 mRNA levels to the difference in phosphate concentration in our two media, it is also possible that the lack of other dialyzable factors from serum may be, at least in part, responsible for the differing receptor levels using this material.

The question of whether PO_4-free medium improves transduction efficiency in human CD34+ cells remains unanswered. With our finding of decreased viability of CD34+ cells during phosphate deprivation, we anticipate a trade-off between higher transduction efficiency and decreased cell viability. In potential future studies, there are at least two approaches to exposing cells to low-PO_4 conditions without significantly lowering their survival in vitro. It may be possible to use less severe phosphate deprivation, perhaps 50% of normal PO_4 concentration, which might still result in maximal up-regulation of GLVR-2 without adversely affecting cell viability. Alternatively, a shorter exposure time to PO_4-free media, 24 hr, for example, may achieve full GLVR-2 up-regulation without decreasing cell viability. We are encouraged by our finding that GLVR-2 expression in human CD34+ hematopoietic progenitor cells can be enhanced by culture in PO_4-free medium. Further studies will hopefully clarify the potential role of up-regulating GLVR-2 expression in retroviral mediated gene transfer into CD34+ cells, and especially into subsets of these

cells such as CD34+CD38− cells, known to be enriched for hematopoietic stem-cell activity.

Alternatively, it may be necessary to target receptors other than GLVR-2 on CD34+ cells. One such candidate receptor is that targeted by gibbon ape leukemia virus (designated Glvr-1 in rodents and GLVR-1 in humans) [32]. High levels of GLVR-1 are present on EBV immortalized B-cells from patients with leukocyte adhesion deficiency [33]; this correlated with higher transduction efficiencies using a retroviral vector (PG13) targeting this receptor, compared with an amphotropic retroviral vector (PA317) which targets GLVR-2. Similarly, GLVR-1 levels in human lymphocytes were found to be higher than GLVR-2 levels and correlated with improved transduction by using a retroviral vector which targets GLVR-1 rather than GLVR-2 [27]. However, in recent primate studies, GLVR-1-targeted CD34+ cells appear to have a similar transduction efficiency to that observed when GLVR-2 is used [34].

In addition, it may be possible to use the vesicular stomatitis virus G protein instead of the retroviral amphotropic envelopes to transduce CD34+ cells. VSV-G does not target a specific receptor, transduces certain target cells efficiently, and viruses containing this protein can easily be concentrated for higher titers [35,36]. In addition, hybrid envelopes targeting other receptors and/or lentiviral vectors may be useful in solving the important problem of high-level transduction necessary to achieve the goal of hematopoietic cell gene therapy [37–39].

CONCLUSIONS

In summary, we have used competitive RT PCR to quantitate the expression of the Moloney amphotropic retroviral receptor—pit-2 or GLVR-2—a sodium-dependent phosphate transporter on CD34+ cells from human peripheral blood harvests. GLVR-2 mRNA expression is up-regulated 2–3-fold after 3 days in culture with IL-3, IL-6, and SCF. In addition, there is a further 2-fold increase in GLVR-2 mRNA when phosphate-free medium is used. These data suggest that GLVR-2 expression can be increased significantly on CD34+ cells, and this may lead to improved retroviral gene transfer into human hematopoietic cells.

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